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14. ABSTRACT

The major goal of this project is to examine the efficacy of oral administration of curcumin (CUR) encapsulated biodegradable polymer nanosystems (nCUR) for alleviating cognitive, memory and mood impairments in a rat model of gulf war illness (GWI). Specific Aim 1 studies involve quantification of the efficacy of oral administration of different doses of nCUR for improving neurogenesis and suppressing inflammation and oxidative stress in the hippocampus. Specific Aim 2 studies will examine whether an apt dose of nCUR treatment (based on Specific Aim 1 studies) is efficient for alleviating cognitive, memory and mood impairments in GWI-rats, when treatment is commenced at an extended time-point (8-months) after exposure to GWIR chemicals and stress. During the past year, a set of experiments related to Specific Aim 1 studies was performed: (1) Exposure of rats to GWI-related chemicals and moderate stress. (2) Oral administration of different doses of nCUR (10, 20 or 40 mg/Kg, thrice weekly for 6 weeks), commencing 2-months after the exposure to GWI-related chemicals and stress. (3) Quantification of hippocampal neurogenesis. (4) Characterization of hippocampal inflammation via analyses of activated microglia. The results collected so far suggest that administration of a lower dose of nCUR (10 mg/Kg) is efficacious for greatly enhancing hippocampal neurogenesis than higher doses (20 or 40 mg/Kg) in GWI-rats. However, all three doses of nCUR appeared to reduce the occurrence of activated microglia in the hippocampus. Overall, the results suggested that 10mg\Kg dose of nCUR is an efficient dose for both enhancing neurogenesis and suppressing inflammation in the hippocampus of GWI rats.

15. SUBJECT TERMS

Curcumin nanoparticles, DEET, Gulf war illness, hippocampus, neurogenesis, Memory and Mood function, neuroinflammation, oxidative stress, Permethrin, and Pyridostigmine bromide

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1. INTRODUCTION

Gulf war illness (GWI) is a chronic multi-symptom health problem, which afflicts ~30% of veterans who served in the Persian Gulf War-1 (PGW-1). Brain dysfunction, typified by cognitive, memory and mood impairments, is one of the major health issues in GWI. While the precise etiology of GWI is unknown, several suspected causes have been proposed. Among these, the hypothesis that GWI is linked to a combination of exposures encountered by service personnel during the war has received much attention. First, veterans who were stationed in the battlefield areas believed to have consumed pills of pyridostigmine bromide (PB) during the war. PB was employed as a prophylactic treatment to protect against a possible attack with nerve gas agents. Second, preparations for the PGW-1 comprised measures to offset infectious diseases transmitted by insects/ticks. The measures included the use of pesticides for the area protection and insect repellants on the skin and uniforms. The pesticides included the insecticide permethrin (PM) and the insect repellant DEET. Thus, in view of the exposure of service personnel to the above GWI-related (GWIR) chemicals and war related stress, it is hypothesized that the neurological symptoms displayed by a significant number of PGW-1 veterans are due to synergistic interaction of PB with pesticides PM and DEET and/or stress. This chemical exposure hypothesis is also supported by Research advisory committee (RAC) reports on GWI that the overall prevalence of GWI is greater in veterans who used higher amounts of pesticides than veterans who had limited exposure to pesticides during the PGW-1. Consistent with this, studies in our laboratory using rat models have shown that a combined exposure to low doses of chemicals PB, PM and DEET with or without mild stress for four weeks causes dysfunction of the hippocampus, a region of the brain vital for cognitive function and mood. Hippocampal dysfunction was typified by learning, memory and mood impairments. Importantly, these changes were associated with greatly waned neurogenesis, chronic inflammation (evidenced by the presence of activated microglia and hypertrophy of astrocytes) and increased oxidative stress in the hippocampus. Decreased neurogenesis, chronic inflammation and elevated oxidative stress can adversely affect cognitive, memory and mood function. From this perspective, drugs capable of enhancing neurogenesis, and/or reducing inflammation and oxidative stress in the hippocampus may reverse cognitive, memory and mood impairments observed in GWI. This project investigates the efficacy of oral administration of curcumin nanoparticles (a drug having neurogenic, antioxidant and anti-inflammatory properties) for easing cognitive, memory and mood impairments in a rat model of GWI.

2. KEYWORDS

Anxiety
Curcumin Nanoparticles
DEET
Gulf war illness
Hippocampus
Neurogenesis
Memory dysfunction
Mood dysfunction
Neuroinflammation
Oxidative Stress
Permethrin
Pyridostigmine
bromide

3. ACCOMPLISHMENTS

3.1. Major Goals:

The major goal of this project is to examine the efficacy of oral administration of curcumin (CUR) encapsulated biodegradable polymer nanosystems (nCUR) for alleviating cognitive, memory and mood impairments in a rat model of gulf war illness (GWI). Specific Aim 1 studies involve quantification of the efficacy of oral administration of different doses of nCUR for improving neurogenesis and suppressing inflammation and oxidative stress in the hippocampus. Specific Aim 2 studies will examine whether an apt dose of nCUR treatment (based on Specific Aim 1 studies) is efficient for alleviating cognitive, memory and mood impairments in GWI-rats, when treatment is commenced at an extended time-point (8-months) after exposure to GWIR chemicals and stress. It is envisaged that improved cognitive, memory and mood function in GWI-rats with nCUR administration will occur via improvements in hippocampal neurogenesis, and suppression of inflammation and oxidative stress. The goal is to test the efficacy of nCUR to alleviate the neurobehavioral impairments seen in the chronic phase of GWI. Eight months waiting period after exposure is equivalent to ~23-24 years survival period after exposure in humans. This extended waiting period is needed to simulate the scenario in patients afflicted with GWI.

3.2. Studies Accomplished During the Past Year:

3.2.1. Specific Activities:

The following narrative describes studies accomplished.

3.2.1.1. Animal numbers, survival and tissue harvesting:

(1) A total of 104 rats have been purchased so far in 2 different cohorts.

(a) The first cohort comprised 40 animals at the start of experiments. From these, 39 animals reached the endpoint of experiments; one animal was found dead during the two- month waiting period between the exposure of animals (to Gulf war illness related (GWIR) chemicals and 15 minutes of restraint stress for 28 days) and the commencement of nCUR treatment. The brain tissues from 39 animals have been harvested for histological studies, which belong to the following groups (n=7-8/group):

Group 1: GWI-rats receiving nCUR at 10mg/Kg,
Group 2: GWI-rats receiving nCUR at 20mg/Kg,
Group 3: GWI-rats receiving nCUR at 40mg/Kg,
Group 4: GWI-rats receiving empty nanoparticles (VEH),
Group 5: Age-matched naïve control rats

(b) The second cohort comprised 64 animals. These animals are assigned to Aim 2 experiments. Experiments have commenced for this cohort of animals.

(1) GWI-nCUR, n=16
(2) GWI-empty nanoparticles, n=16
(3) GWI alone, n=16
(4) Naive control animals, n =16

3.2.1.2. Time-line of various procedures for animals in cohort 1 (Aim 1 studies):

(i) Exposure period to GWIR-chemicals and stress:	28 days (daily)
(ii) Survival period between exposure and treatment:	2 months
(iii) nCUR or VEH treatment period:	8 weeks (3 times/week)
(iv) 5'-bromodeoxyuridine (BrdU) injection period:	5 days (in the 5th week of nCUR treatment)
(iv) Euthanasia and Tissue harvesting:	After 8 weeks of nCUR/VEH treatment

These animals have been perfused and tissues harvested.

3.2.1.3. Time-line of various procedures planned for animals in cohort 2 (Aim 2 studies):

(i) Exposure period to GWIR-chemicals and stress:	28 days (daily)
(ii) Survival period between exposure and treatment:	8 months
(iii) nCUR or VEH treatment period:	8 weeks (3 times/week)
(iv) 5'-bromodeoxyuridine (BrdU) injection period:	5 days (in the 5th week of nCUR treatment)
(iv) Cognitive and mood function tests	Starts from 5th week of nCUR treatment
(v) Euthanasia and Tissue harvesting:	After 8 weeks of treatment

These animals are currently in 8 months waiting period after exposure to GWIR-chemicals and stress.

3.2.1.4. Brief description of procedures performed so far:

(a) Exposure of animals to GWIR-chemicals and stress: Animals were exposed daily to the following chemicals for 28 days: Pyridostigmine bromide (PB) at 2 mg/kg/day (via oral gavage), DEET at 60 mg/kg/day (via dermal application) and Permethrin at 0.2 mg/kg/day (via dermal application). In addition, animals were subjected daily to 15 minutes of restraint stress using rat restrainers during the above 28-day period.

(b) Survival period between exposure and treatment: Following the exposure to GWIR-chemicals and stress, animals were maintained in the vivarium for 2-8 months in regular cages (two per cage) with ad libitum access to food and water.

(c) Administration of nCUR or VEH (Cohort 1): Treatment was given for 8 weeks for animals in cohort 1 (3 times/week) via oral gavage, commencing 2 months after exposure to GWIR- chemicals and stress. The doses of nCUR employed were 10 mg/Kg, 20 mg/Kg and 40 mg/Kg.

(d) BrdU injections: Subgroups of rats from all groups received BrdU injections in the 5th week of nCUR or VEH treatment daily for 5 days at a dose of 100 mg/Kg/day.

(f) Euthanasia and tissue harvesting: Animals belonging to cohort 1 were deeply anesthetized with isoflurane in a small chamber, until the animal ceased respiration. Deeply anesthetized animals were perfused through the heart with 4% paraformaldehyde solution. Fixed tissues were harvested for histological studies.

(g) Immunohistochemical studies: Fixed brain tissues obtained from animals belonging to cohort #1 were processed for cryostat sectioning. Serial sections (every 15th or 20th) through the entire hippocampus were processed for the following staining. (i) BrdU immunostaining, for detection of newly born cells in the hippocampus. (ii) BrdU and neuron-specific nuclear antigen (NeuN) dual immunofluorescence and confocal microscopy, for assessing neuronal differentiation of newly born cells. (iii) Doublecortin (DCX) immunostaining, for measuring the ongoing status of hippocampal neurogenesis. (iv) Immunostaining for IBA-1 (a marker of all microglia), ED-1 (a marker of activated microglia), glial fibrillary acidic protein, (GFAP, a marker of astrocytes), and IBA-1 and ED-1 dual immunofluorescence and confocal microscopy, for assessing neuroinflammation. The BrdU+ cells, DCX+ neurons in the hippocampal subgranular zone-granule cell layer (SGZ-GCL) were quantified using stereology. Dual labeled cells (i.e. cells positive for BrdU and NeuN and cells positive for IBA-1 and ED-1) were quantified via Z-section analyses in a confocal microscope.

3.2.2. Progress details:

3.2.2.1. Four weeks of nCUR treatment at 10 mg/Kg to GWI-rats improved net hippocampal neurogenesis to age-matched naïve control levels: We quantified net hippocampal neurogenesis in all experimental groups through daily injections of BrdU, a thymidine analog that labels all proliferating cells in the S-phase of cell cycle and hence serves as a birth-dating marker. GWI rats received daily i.p. injections of BrdU for 5 days in the 5th week of nCUR or VEH treatment, which labeled cells that were added to the neurogenic region (SGZ-GCL) of the hippocampus in the 5th week of treatment.

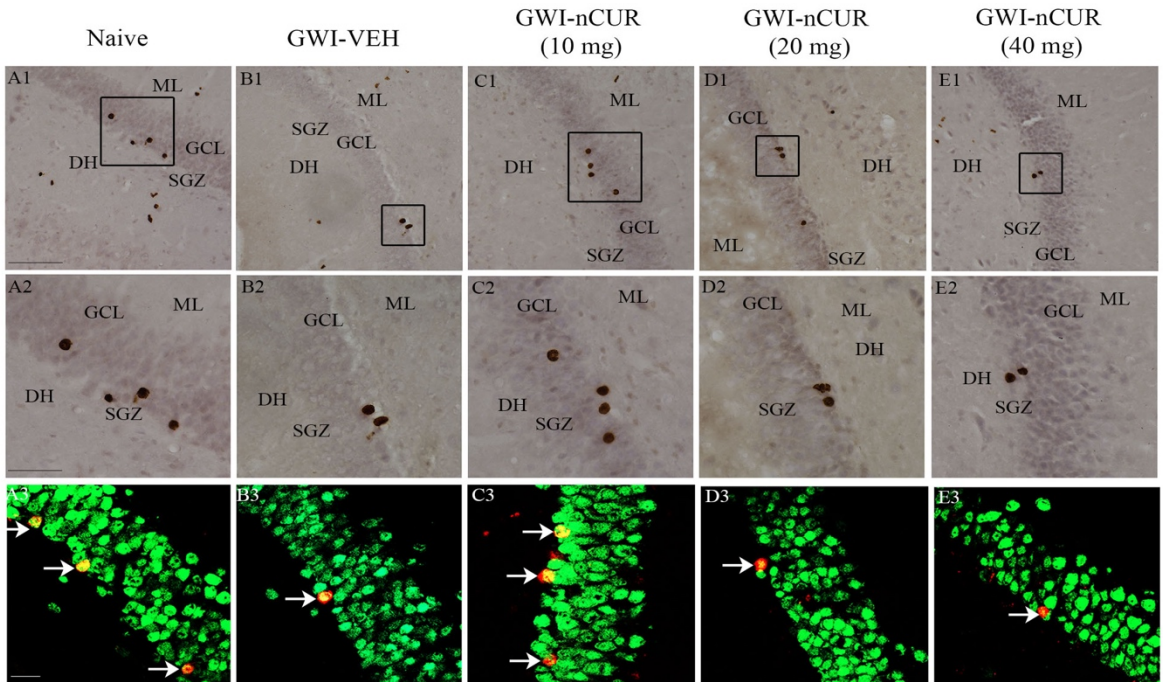

Figure 1 *- Figures in the top panel illustrate BrdU+ cells in the SGZ-GCL of the hippocampus from a naive control rat (A1), and GWI rats receiving VEH (B1) or nCUR at 10 mg/Kg (C1), 20mg/Kg (D1) or 40mg/Kg (E1). DH, dentate hilus; GCL, granule cell layer; ML, molecular layer; SGZ, subgranular zone.*

Figure 1 illustrates the distribution of BrdU+ cells in the SGZ-GCL ~3.5 weeks after

BrdU injections. Greater numbers of BrdU+ cells were seen in GWI-rats receiving nCUR at 10mg/Kg but not in GWI-rats receiving 20 or 40 mg/Kg doses. (Fig. 1). BrdU-NeuN dual immunofluorescence and Z-section analysis in a confocal microscope revealed neuronal differentiation of significant percentages of BrdU+ cells in all groups. Examples of newly born cells that expressed BrdU and NeuN are illustrated in Fig. 1 (A3, B3, C3, D3, E3).

Stereological counting of BrdU+ cells (Fig. 2, left panel) and measurement of percentages of BrdU+ cells expressing the mature neuronal marker NeuN (Fig. 2, middle panel) in the SGZ-GCL facilitated the quantification of net hippocampal neurogenesis (Fig. 2, right panel). Decreased addition of newly born cells (BrdU+ cells) was seen in GWI rats receiving VEH, in comparison to naive control rats (Fig. 2, left panel), which is consistent with our previous studies. Remarkably, 4 weeks of nCUR treatment at a lower dose (10mg/Kg) normalized the addition of newly born cells to levels observed in age-matched naïve control animals. However, such restorative effects were not seen with the administration of nCUR at 20 mg/Kg or 40 mg/Kg (Fig. 2, left panel). Numbers of BrdU+ cells in these higher dose nCUR groups were comparable to numbers seen in GWI-rats receiving VEH.

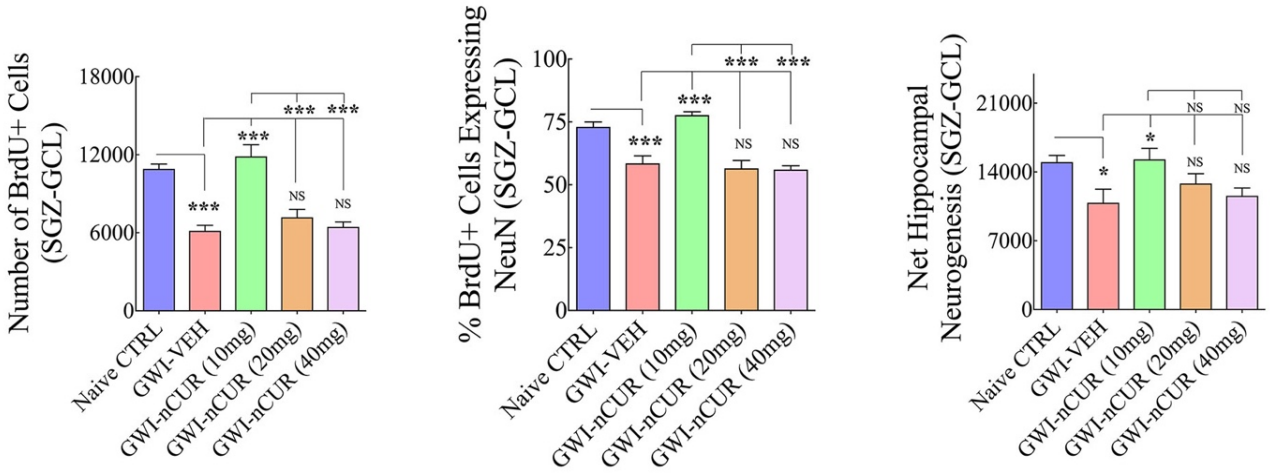

Figure 2: *Bar charts compare numbers of BrdU+ newly born cells (left panel), percentages of BrdU+ cells expressing NeuN (middle panel) and net hippocampal neurogenesis in the SGZ-GCL of the hippocampus between different groups of rats (n=6-7/group). *, p<0.05; ***, p<0.001; NS, not significant.*

Quantification of the neuronal differentiation of newly born cells showed a similar trend. The extent of neuronal differentiation was greater in naïve control rats and GWI-rats receiving 10mg/Kg dose of nCUR (Fig. 2, middle panel). Measurement of net hippocampal neurogenesis revealed a decline in GWI-rats receiving VEH but complete recovery in GWI-rats receiving 10 mg/Kg nCUR (Fig. 2, right panel). However, GWI-rats receiving nCUR at higher doses displayed similar levels of neurogenesis as GWI rats receiving VEH (Fig. 2, right panel). **Thus, four weeks of nCUR treatment at 10 mg/Kg to GWI-rats revitalized hippocampal neurogenesis, a form of hippocampal plasticity important for functions such as learning, memory and mood.** This lower dose of nCUR treatment enhanced not only the production of new cells but also the neuronal differentiation of newly born cells in the neurogenic region, which resulted in enhanced net hippocampal neurogenesis. *It is unclear why higher doses of nCUR failed to improve hippocampal neurogenesis. It may be*

that nCUR at higher doses interferes with the survival of newly born cells and neurons. Additional studies are needed to determine this possibility.

3.2.2.2. Eight weeks of nCUR treatment at 10 mg/Kg to GWI-rats greatly enhanced the production of newly born neurons: We investigated newly born neurons expressing doublecortin (DCX) in the SGZ-GCL of the hippocampus in all groups (Fig. 3). DCX, a microtubule associated protein expressed in immature neurons, serves as an excellent marker of newly born neurons in the SGZ-GCL of the hippocampus. Since DCX expression in newly born neurons persists for about two weeks in rats, counting of DCX+ neurons in the SGZ-GCL provides information on the extent of addition of newly born neurons that occurred over two weeks preceding euthanasia. Figure 3 illustrates examples of DCX+ newly born neuron density in the SGZ-GCL of animals from different groups. Interestingly, GWI-rats receiving nCUR at 10 mg/Kg displayed a greater density of DCX+ neurons than GWI rats receiving VEH as well as naïve control animals (Fig. 3).

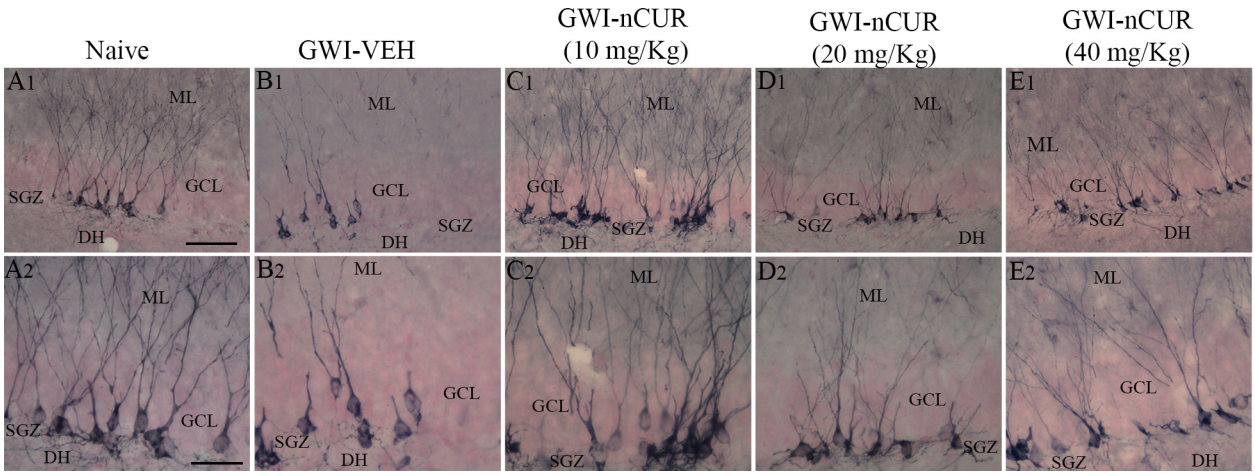

Figure 3 *- Figures in A1-E2 illustrate DCX+ newly born neurons in the SGZ-GCL of the hippocampus from a naive control rat (A1), and GWI-rats receiving VEH (B1) or nCUR at 10 mg/Kg (C1), 20mg/Kg (D1) or 40mg/Kg (E1). A2, B2, C2, D2 and E2 are magnified views of regions from A1, B1, C1, D1 and E1. DH, dentate hilus; GCL, granule cell layer; ML, molecular layer; SGZ, subgranular zone.*

Stereological quantification of the numbers of DCX+ immature neurons revealed decreased addition of newly born neurons in the hippocampus of GWI rats receiving VEH, in comparison to naive control rats (Fig. 4). In contrast, GWI-rats receiving a lower dose of nCUR (10 mg/Kg) displayed enhanced neurogenesis, in comparison to both GWI rats receiving VEH and age-matched naïve control rats (Fig. 4). GWI-rats receiving nCUR at 20 mg/Kg showed similar numbers of newly born neurons as naïve control animals (Fig. 4). On the other hand, addition of newly born neurons in GWI-rats receiving nCUR at 40mg/Kg was improved in comparison to GWI-rats receiving vehicle but remained lower than numbers seen in naïve control animals (Fig. 4).

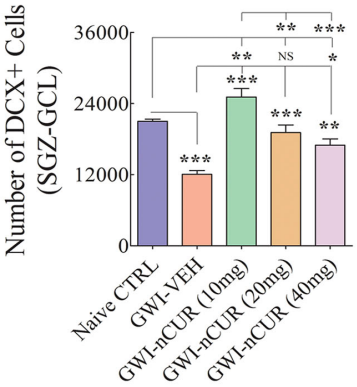

Figure 4 *– The bar chart compares numbers of DCX+ newly born neurons in the SGZ-GCL of the hippocampus between different groups of rats (n=6-7/group). *, p<0.05; **, p<0.01; ***, p<0.001.*

Thus, 8 weeks of nCUR treatment at a lower dose (10mg/Kg) is efficient for greatly enhancing the production of new neurons in the hippocampus of GWI-rats. Administration of nCUR at higher doses (20 or 40 mg) was not as effective as the lower dose of nCUR but

some increases were seen in comparison to GWI rats receiving VEH. It remains to be determined why a lower dose of nCUR is more effective than higher dose of nCUR however.

3.2.2.2. Eight weeks of nCUR treatment to GWI-rats reduced the numbers of activated microglia in the hippocampus at all doses: We investigated microglia in all GWI groups using dual immunofluorescence for IBA-1 (a marker of all microglia) and ED-1 (CD68; a marker of activated microglia). Microglial cells expressing both IBA-1 and ED-1 were identified through Z-section analyses in a confocal microscope (Fig. 5). Quantification revealed that 8 weeks of nCUR treatment at all doses (10, 20, 40 mg/Kg) reduced the percentages of IBA-1+ cells expressing ED-1 (Fig. 5, bar chart on the right). Since ED-1 is a specific marker of activated microglia, these results imply that nCUR treatment is efficient for reducing the extent of inflammation in the hippocampus of GWI-rats.

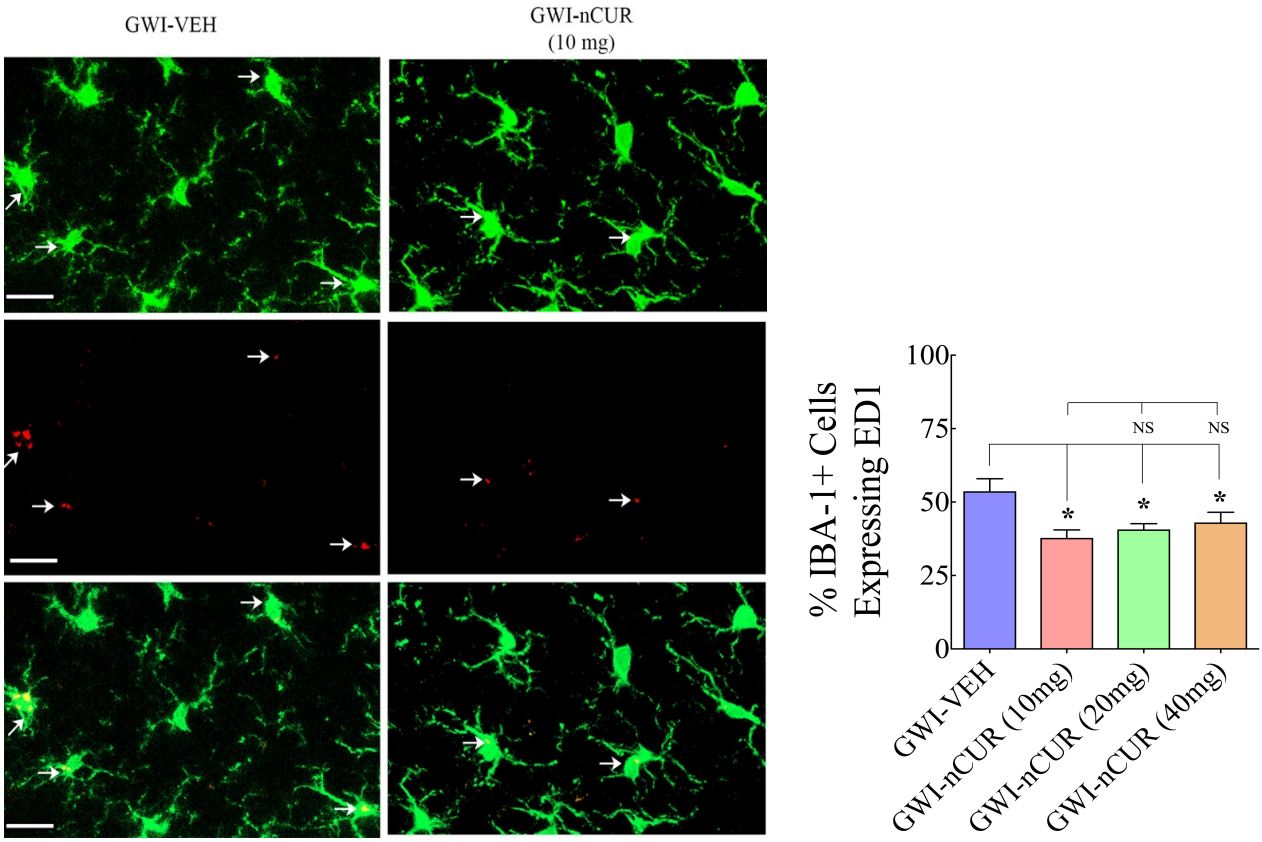

Figure 5: *Images show IBA-1+ microglia (green, top panel) expressing ED-1 (red, middle panel) in the hippocampus of a GWI-rat receiving vehicle (left panels) and a GWI-rat receiving nCUR (right panels). The lower panel illustrates merged images. Arrows denote activated microglia expressing both IBA-1 and ED-1. The bar chart on the right compares the numbers of activated microglia between different groups. Note that, numbers of activated microglia are reduced in GWI-rat groups receiving different doses of nCUR, in comparison to GWI-rats receiving vehicle. *, p<0.05.*

3.3. Opportunities for Training and Professional Development

Nothing to Report

3.4. Dissemination of Results to Communities of Interest:

Nothing to Report

3.5. Plans for the Next Reporting Period:

In the coming year, we will continue studies related to Specific Aims 1 and 2.

(A) Using tissues that are already harvested (Aim 1 studies), we will perform:

(i) Analyses of IBA-1+ microglia (all microglia) and counts of ED-1+ microglia (activated microglia) in the hippocampus to assess inflammation.

(ii) Quantification of the area fraction of GFAP+ astrocyte cell bodies and processes using J imaging in different subfields of the hippocampus to assess inflammation.

(iii) Analyses of the proliferation of neural stem cells (NSCs) in the SGZ using Ki-67 and Sox-2 dual immunofluorescence and confocal microscopic analyses.

(B) We will continue the scheduled experiments for animals (n=64) assigned to Specific Aim 2 (Cohort 2). These animals are currently in 8-month waiting period after the exposure to GWIR-chemicals and stress. The remaining experiments will involve:

(i) nCUR (or vehicle) treatment for 8 weeks (thrice/week), commencing eight months after the exposure to GWIR-chemicals and stress.

(ii) Injections of BrdU (once daily for 5 days at 100 mg/Kg). This will be done in the 5th week of nCUR treatment regimen.

(iii) Analyses of cognitive, memory and mood function using a battery of neurobehavioral tests, commencing in the 6^{th} week of nCUR or VEH treatment (n=16/group, total, 64 rats in 4 groups).

(iv) Euthanasia: 50% of animals in all groups will be perfused with 4% paraformaldehyde for histological studies, and another 50% of animals will be euthanized to harvest fresh brain tissues for biochemical and molecular biological studies. The histological and molecular biological studies will commence in year 2 but will be completed in year 3 of the project.

(C) We will generate another cohort of GWI rats for completing experiments of Specific Aim 1 (n=48). These animals will be treated with different doses of nCUR (10, 20 or 40 ng/Kg) or VEH, commencing 2 months after exposure to GWIR-chemicals and stress. Fresh brain tissues will be harvested for biochemical and molecular biological studies following 8 weeks of nCUR or VEH treatment. The biochemical and molecular biological studies will commence in year 2 but will be completed in year 3 of the project.

4. IMPACT:

The results collected from studies conducted so far suggest that oral administration of nCUR at a lower dose (10 mg/Kg) is most efficacious for enhancing neurogenesis in GWI rats, when administration commenced 2 months after the exposure to GWIR-chemicals and stress. However, all dose of nCUR tested (10, 20, 40 mg/Kg) were effective in reducing the occurrence of activated microglia.

5. CHANGES AND PROBLEMS:

(i) Changes in approach:

No changes in approach will be required.

(ii) Actual or Anticipated Problems or Delays and Plans to Resolve them:

Nothing to Report:

(iii) Changes that had a significant impact on expenditures:

Nothing to Report

(iv) Significant Changes in the use of vertebrate animals or biohazards:

Nothing to Report

(v) Significant Changes in the Care of Vertebrate Animals:

Nothing to Report

6. PRODUCTS:

Publications:

Nothing to Report

7. PARTICIPANTS AND OTHER COLLABORATIVE ORGANIZATIONS

The following research staff members from PI's laboratory were compensated from this grant (for the percentage of effort contributed to this project)

Personnel	Role	Percent Effort
Ashok K. Shetty, PhD	Principal Investigator	10%
S. Attaluri, M. Pharm	Research Assistant	90%
Bing Shuai, M.D.	Senior Research Associate	50%
Meenakshi Arora, Ph.D.	Postdoctoral Research Fellow	50%
Ravi Majeti, Ph.D.	Co-Investigator	10%

Other Collaborators: None

Changes in active other support of the PI or Key Personnel:

There will be a change in the percentage effort of Ms. Sahithi Attaluri (Research Assistant) in the second year of this project. Her effort will decrease to 50% (from the current 90%). A Senior Research Associate (Laila Dayani, 25% effort) and a Research Assistant (Eeshika Mitra, 25% effort) will work part-time for this project in the second year.

Other Organizations Involved in this Project: None

8. SPECIAL REPORTING REQUIREMENTS

None

9. APPENDICES

None